Chromosome Aberrations as a Measure of Mutagenesis: Comparisons *in Vitro* and *in Vivo* and in Somatic and Germ Cells*

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Structural alterations to chromosomes constitute a significant portion of the genetic damage produced by most mutagenic agents. The pioneering experiments of Muller (*1*), in the case of ionizing radiations, and of Auerbach (*2*), in the case of nitrogen mustards, showed that chromosome aberrations are, quantitatively, a significant class of mutational events for both physical and chemical mutagens.

The ultimate goal in any mutagen screening program is to be able to test potential mutagens directly on human genetic material or to extrapolate to man from other test systems with confidence. At present the easiest way to attain that goal is to use chromosomal aberrations as an index of mutational damage. It is quite simple to obtain a large number of homogeneous human cells, treat them with the agent in question, analyze the chromosomes at the subsequent mitosis, and, if desired, compare the results to identical studies done on other mammalian species.

The technique employed in this instance is short-term tissue culture of peripheral leukocytes (*3*). The procedure is technically simple, but it presents particular uncertainties and difficulties in interpreting the data. The experiments on human cells must be done *in vitro*, and the question arises as to whether the response is quantitatively and qualitatively the same as that *in vivo*. Second, the experiments are done on somatic cells, and the effect on the genetically important germ cells may be quite different. Third, even if the experiments are done on laboratory animals, where the germ ecIls can be treated *in vivo*, the problem of differential cell stage sensitivity (*4,5*) and interspecific variations in sensitivity (*6*) must be accounted for.

The present paper is addressed to those problems. The majority of the data are derived from experiments utilizing low-LET radiations as the mutagenic agent, although some data are presented from studies of chemical mutagens.

Comparisons of Aberration Production in Vivo and in Vitro

The most extensive quantitative data on the production of damage to human genetic material have come from studies on the induction of chromosome aberrations in peri-

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pheral leukocytes by ionizing radiations. With a few notable exceptions of accidental and therapeutic exposures, the data have been derived from *in vitro* experimentation.

McFee et al. (*7*) first compared *in vivo* and *in vitro* production of chromosomal aberrations by ionizing radiation in their study on swine leukocytes irradiated with 14-MeV neutrons. Their data showed a significant difference in the quantitative yield, depending upon whether the exposure was given *in vivo* or *in vitro*. The data, however, are difficult to interpret because of dosimetric problems arising from tissue shielding and dose attenuation. The authors made calculations to account for these difficulties (*7*), but the *in vivo* doses carry a large standard error due to blood distribution through the body. Subsequent to that study, we conducted extensive comparisons of aberration production *in vivo* and *in vitro* by low-LET radiations in peripheral leukocytes of three mammalian species: *Mus musculus (6), Cricetulus griseus (8),* and *Saguinus fuscicollis (9)*. All three species are small, so the problem of dose attenuation was minimal. The results of those experiments are summarized in Table 1.

The data lead us to conclude that when the cells receive comparable doses of ionizing radiation the chromosome damage sustained is quantitatively and qualitatively the same *in vivo* and *in vitro*.

The problems encountered in making similar comparisons for chemical mutagens are considerably more complex. First, administration of equivalent doses on a weight-volume basis to the target cells is very difficult. Second, if it is assumed that equivalent doses are administered to the target cells, consideration must be given to the fact that for *in vitro* testing the cells are directly exposed to the compound, whereas in *in vivo* testing the compound is being carried to the target cells by the circulatory system and must cross the blood–tissue barrier. Third, the compound may be detoxified *in vivo* by tissues other than the target cells. And fourth, some metabolites of the compound may be mutagenic themselves. These problems make the *in vitro* testing of compounds for *in vivo* extrapolation virtually impossible.

There are several examples where the above problems are manifested in experimental testing. Brock and Hohorst (*10*) have shown that cyclophosphamide induces chromosomal aberrations *in vivo* but not *in vitro*. The work of Schmid et al. (*11*) and Arakaki and Schmid (*12*) shows that, to produce equivalent degrees of chromosome damage in Chinese hamster bone marrow cells treated *in vivo* and Chinese hamster fibroblast cells treated *in vitro*, the dose of Trenimon administered *in vivo* has to be 100 times higher than the dose *in vitro*. The data of Arakaki and Schmid (*12*) also show that the amount of damage depends on the cell type used in the *in vitro* experiments.

We have done similar experiments with an alkylating agent. Human leukocytes were

Table 1. Frequencies of centric rings plus dicentrics observed in the peripheral leukocytes of *M. musculus*, *C. griseus*, and *S. fisciollis* after *in vivo* and *in vitro* irradiation.

	Frequency, %					
	M. musculus		*C. griseus*		*S. fuscicollis* [a]	
Dose, R	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
0	0.0	0.3 ± 0.3	0.0	0.0	0.0	0.0
50	4.3 ± 1.2	2.3 ± 0.9	2.7 ± 1.0	3.5 ± 1.1	—	—
100	7.3 ± 1.6	6.7 ± 1.5	7.0 ± 1.5	8.0 ± 1.6	13.7 ± 1.2	13.3 ± 2.1
200	19.7 ± 2.6	14.3 ± 2.2	18.6 ± 2.3	21.0 ± 2.7	40.0 ± 2.1	33.3 ± 3.3
300	27.4 ± 4.3	31.3 ± 3.2	—	38.0 ± 3.6	70.7 ± 4.9	68.7 ± 4.8
400	51.7 ± 4.2	65.0 ± 8.0	—	63.3 ± 4.6	94.4 ± 6.1	98.3 ± 5.7

[a] Irradiation was with ^{60}Co γ-rays at a dose rate of 3.7 R/min.

exposed to methyl methanesulfonate (MMS) either *in vitro* or in a simulated *in vivo* environment consisting of diffusion chambers implanted in mice (*13*). The mice were injected either intraperitoneally or intravenously with 130 mg/kg of MMS 12 or 24 hr before the diffusion chambers were removed and the human leukocytes were prepared for chromosome analysis. The *in vitro* regimen consisted of treating short-term cultures of peripheral leukocytes with $1.2 \times 10^{-4}M$ (0.013 mg/ml) MMS for 2 hr beginning 6, 12, or 18 hr before the cultures were fixed for chromosome analysis. The *in vitro* dose was thus only 1/10 the *in vivo* dose. The cells were also pulse-labeled with tritiated thymidine so that cells replicating their DNA at the onset of the *in vitro* exposure could be distinguished from those that were not.

The results of the experiment are summarized in Table 2. Since there was no significant difference in the data for the 6- and 12-hr treatments in the *in vitro* experiment, or between intravenous and intraperitoneal administration of the MMS in the *in vivo* experiment, these respective groups are pooled in Table 2. The data indicate two things: (1) cells replicating their DNA are approximately five times as sensitive as cells in G_1 or G_2, and (2) there is an "apparent" difference of one to two orders of magnitude between the *in vivo* and *in vitro* sensitivities. The quantitative difference between the *in vivo* and *in vitro* responses is difficult to interpret. It may reflect a failure of the MMS to penetrate the Millipore filter used in constructing the diffusion chambers, detoxification by the host animal, or inadequate sample size of sensitive (i.e., S-stage) cells examined in each instance; or it may indicate a true difference in response by the same cell type under two different sets of conditions. It is obvious, however, that the problems presented to the study of mutagenesis by chemicals are much more complex than those encountered in radiation studies.

Interspecific Comparisons

Before quantitative data derived in mutagenesis testing on laboratory animals can be used to make meaningful predictive statements concerning man, a "bridge" of information is needed between the two species. At present, chromosome aberration studies appear to offer the greatest possibility for establishing that "bridge", simply because there is already a great deal of information and because the current techniques lend themselves to rapid accumulation of large amounts of data.

We have recently finished an interspecific comparison of the radio-sensitivities of the peripheral leukocytes of several mammalian species (*6*). In those experiments we irradiated freshly drawn blood, or the animal under study, with x-rays or γ-rays at a high dose rate. The leukocytes were then cultured in various ways, and the resulting first mitotic divisions were analyzed for chromosome-type aberrations. The aberration types analyzed were asymmetrical interchanges (dicentrics), asymmetrical in-

Table 2. Frequencies of various types of chromatid aberrations in MMS-treated human leukocytes.

Time, hr [a]	No. cells scored	L, % [b]	U, % [b]	*Deletions, %*		*Exchanges, %*	
				L	U	L	U
In vitro							
6,12	390	33.8	66.2	33.3	5.8	1.5	0
18	168	54.0	46.0	84.6	13.0	117.6	7.8
In vivo							
12	600	—	—	7.0 ± 1.1		2.7 ± 0.7	
24	700	—	—	4.4 ± 0.8		2.0 ± 0.5	

[a] The time corresponds to the time before fixation that MMS was added.
[b] L = labeled, U = unlabeled.

terarm intrachanges (centric rings), and acentric fragments. Dose–response curves were made, and relative yields of dicentrics and acentric fragments presumed to be deletions were compared among species. Several facts were found. The yields of dicentrics increased exponentially with dose for all species (Fig. 1). Although the yields of deletions were relatively constant among species, the yields of dicentrics were higher in the species with higher chromosome arm numbers. The relationship of dicentrics and chromosome arm number was such that a linear fit to the equation, $Y = (N - 1)(bD + cD^2)$ where N equals the "effective" arm number, was fairly good at each dose (Fig. 2). This indicates that the probability of producing an interchange is directly proportional to chromosome arm number, provided DNA content is kept constant. Thus we conclude that man is twice as sensitive as the mouse to the production of interchanges by ionizing radiation. Since there is preliminary evidence (*14,15*) that dicentric yields are indicative of the genetically important reciprocal translocations (symmetrical interchanges), it seems reasonable to assume that translocation yields observed in the mouse are approximately one-

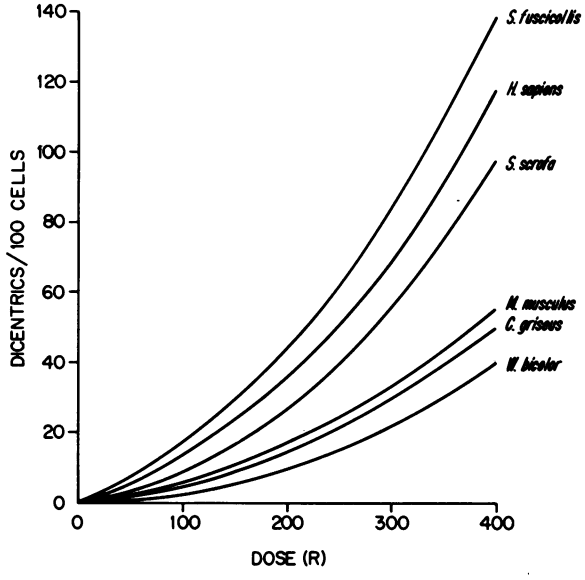

FIGURE 1. Best-estimate regression curves for dicentric production in six mammalian species.

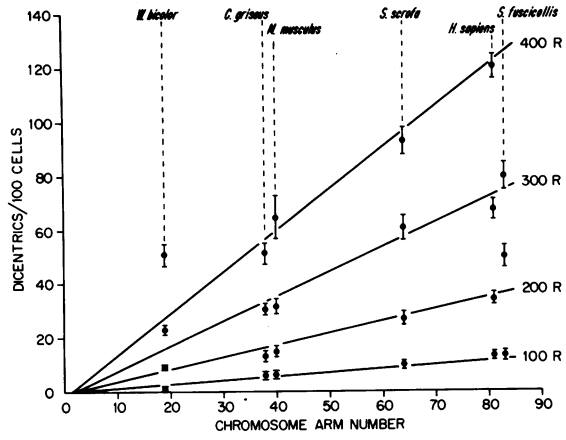

FIGURE 2. Best-estimate regression curves for dicentric yields vs. chromosome arm number in six mammalian species. Observed yields are plotted with standard errors at each of the four doses considered.

half those expected in man at comparable low doses.

Deletion data from the interspecific comparisons in the above experiments (*6*) suggest that mouse and man are equally sensitive to the production of that specific type of genetic damage. Since it has been shown that a significant proportion of the "specific-locus" mutations in the mouse are indeed minute deletions (*16,17*), it might be argued that mouse and man are equally sensitive to such genetic damage.

Interspecific comparisons on the production of chromosomal aberrations by chemical mutagens are still meager. Some comments can be made, however, on the basis of data collected on different species in different laboratories. These comparisons must be tempered with the precaution that different species are very likely to metabolize mutagens in different ways, thus creating the likelihood of diverse quantitative responses. For example, in studies by Legator et al. (*18*) and Brewen et al. (*13*) on the production of chromosome aberrations in the bone marrow of the rat and Chinese hamster, respectively, by cyclamate and its metabolic byproducts, a significant level of chromatid aberrations was found in the rat after repeated doses of cyclamate and cyclohexylamine, whereas no effect was found

in the comparable experiments done on the Chinese hamster. Comparable findings for the cytogenetic effects of mitomycin C on rat bone marrow and mouse spermatogonial cells have been published (*19, 20*).

The issue becomes even more clouded when data derived from other possible mutagens are compared. In some instances, different laboratories obtain significantly different results when the same experimental systems are used. This type of inconsistency makes interspecific comparisons even more difficult. Generally speaking, however, it can be stated that when potent mutagens are tested in various mammalian systems, qualitatively if not quantitatively similar results are obtained.

Comparisons of Aberration Production in Somatic and Germ Cells

Since chromosome aberrations produced in somatic cells in no way pose a genetic hazard to subsequent generations and it is the recoverable damage that is important in a strict genetic sense, it is imperative that we be able to translate data derived from somatic cell studies to the situation in the genetically important germ cells. On the basis of radiation studies, it is obvious that the quantitative level of damage observed in somatic cells is many times greater than that observed in particular stages of spermatogenesis and ultimately recovered in the F_1 progeny of treated individuals. For example, analysis of peripheral leukocytes from a male mouse given 300 R of whole body x-irradiation shows a yield of 31% dicentrics (*6*). In theory, reciprocal translocations should be produced with the same frequency as dicentrics. In the absence of data to the contrary, we assume that this theory is correct. When spermatogonial stem cells are irradiated with the same dose and analyzed at diplotene-diakinesis of the primary spermatocyte stage, the yield of reciprocal translocations is only about 8%. This is only one-fourth the yield that would have been predicted from the leukocyte data. Ford et al. (*21*) found that aproximately one-eighth of the reciprocal translocations scored in primary spermatocytes are recovered in the subsequent F_1. Thus the leukocyte data should be corrected by a factor of 1/32 to arrive at an estimate of genetically recoverable translocation frequency. Why is this so?

Obviously, translocation-bearing cells are lost between the time of treatment (spermatogonia) and the time of cytological analysis (primary spermatocytes). Several suggestions have been offered (*22–25*) to explain this loss: (1) "unstable" (asymmetrical) chromosome aberrations that result in cell death might be present in the same cell as a reciprocal translocation; (2) populations of cells might be either radioresistant to killing and translocation production or radiosensitive to both; and (3) the reciprocal translocations themselves might confer a selective disadvantage to the cells. The first of these alternatives does not adequately explain the low yield of translocations recovered in primary spermatocytes. It has been established that aberrations are distributed in a Poisson fashion after moderate doses of x-irradiation (*26*). Thus there exists a population of cells with no "unstable" aberrations, and within this population the reciprocal translocations have a Poisson distribution, with a mean yield equal to that of the entire population. Hence, if it is assumed that every "unstable" aberration is cell-lethal, the surviving fraction will still have a reciprocal translocation yield equal to the original yield. Cellular radioresistance to both killing and translocation production, and reduced proliferative capacity of translocation-bearing cells, could conceivably reduce the ultimate yield of recovered translocations, but we do not think they can account for a fourfold difference.

There are two other mechanisms that can dramatically reduce the initial yield of reciprocal translocations. For the sake of simplicity let us consider only that class of cells with no "unstable" aberrations and a normal proliferative capacity. Reciprocal translocations will be distributed between

these cells so that the numbers of cells with 0, 1, 2, . . ., etc. will be the terms of a Poisson for the induced mean yield. It has been shown that approximately 10–20% of all translocations are incomplete (*27*), i.e., an acentric fragment is generated by failure of one or more of the chromosome products to rejoin completely. Since loss of large pieces of genetic material is generally cell-lethal (*28,29*), it is expected that this 10–20% of incomplete translocations will be lost through cell death. Since the zero class will not be affected, this will result in a 10–20% reduction in the mean yield. Second, all stages of the cell cycle exist in the spermatogonial germ cells, and consequently chromatid-type aberrations will be formed. Chromatid reciprocal translocations will involve only two of the four chromatid strands, and if random segregation occurs at the next mitotic division four classes of daughter cells are expected to be formed in equal frequency (Fig. 3). Of these four classes one will be normal, one will be a balanced translocation heterozygote, and two will be duplication deficiencies. Cells in the latter two classes will die; consequently, for every chromatid reciprocal translocation that is formed only 0.25 are expected to be recovered. If a cell has two chromatid reciprocal translocations in it, the probability of recovering both of them in a daughter cell will be 1:16, and the probability of recovering at least one will be 1:8.

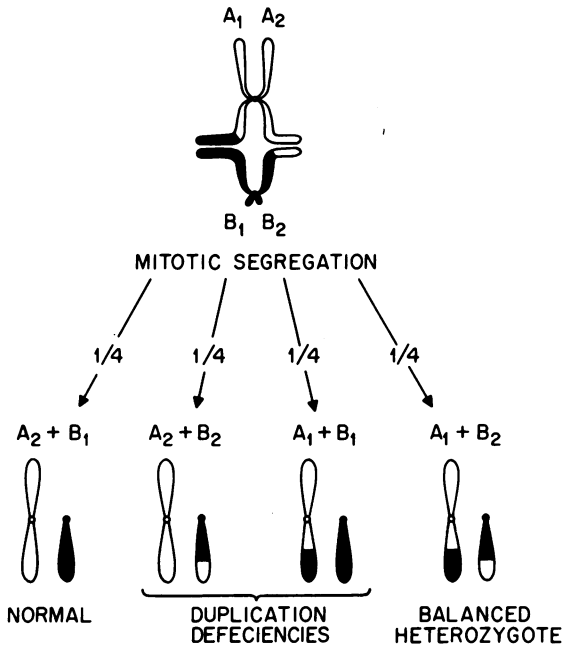

FIGURE 3. Segregation products of a symmetrical chromatid interchange.

It would appear that the low yield of reciprocal translocations observed in primary spermatocytes as opposed to the theoretical yield produced by equal doses in somatic cells can readily be explained by simple cell and chromosome kinetics. The question then arises as to whether these simple explanations can account for some of the apparently inexplicable results obtained with chemical mutagens.

Several papers have reported a complete lack of reciprocal translocations in primary spermatocytes after treatment of spermatogonia with known chemical mutagens (*30–32*). These observations are highly unusual in light of the fact that the same chemicals have been shown to produce dominant lethals and reciprocal translocations when postmeiotic cells are treated (*33–36*), and they are also known to produce chromosome aberrations in the bone marrow and in spermatogonia. Since the aberrations are produced in the spermatogonia, the obvious question is why they are never recovered in the primary spermatocytes. In addition, we also must ask, "How meaningful are tests for chromosome aberration production in bone marrow and spermatogonia by chemicals if the damage is never realized in subsequent generations?"

Most of the chemicals tested to this point are very specific in their time and mode of action (*37*). For the most part the chromosome aberrations are either only produced in those cells that are replicating their DNA or this stage of the cell cycle is much more sensitive than the other stages. When the chemical is administered and a time lag of 12–24 hr ensues before cytological examination, it is next to impossible to identify the stage of the cell cycle the cells were in

at the time of treatment without an additional experimental variable, e.g., tritiated thymidine. Data that are presented as "breaks per cell" then become essentially meaningless, because they do not allow for discrimination of the distribution of the damage on an individual cell basis. Thus, for example, if 200 randomly selected cells are analyzed and the mean yield of exchanges is 0.5 per cell, there is no way to determine whether 100 cells each had one exchange or 50 cells each had two exchanges, or what. Furthermore, only the symmetrical (reciprocal) exchanges are expected to survive, and if these are of the chromatid variety, as is the case when they are induced in the S phase, only a small portion of those are expected to survive the first mitotic division. This argument can be demonstrated more accurately with a theoretical treatment of actual data.

Earlier in the present paper data were presented on the production of chromatid aberrations in human leukocytes by MMS. One culture had been pulse-labeled with tritiated thymidine and treated with MMS 18 hr before metaphase figures were collected. A total of 168 figures were analyzed, and 113 chromatid interchanges were scored, for a yield of 67%. However, when the cells were checked for the presence of label it was found that 91 labeled cells had 107 chromatid exchanges and 77 unlabeled cells had 6 chromatid exchanges, corresponding to yields of 118 and 8%, respectively. If the aberrations in the labeled cells were distributed in a random fashion we would expect half of them to be cell-lethal, asymmetrical types and half to be symmetrical types. Thus the yield of symmetrical exchanges in the labeled cells would be approximately 60% and the proportion of cells with 0, 1, 2, 3, ..., etc. exchanges would be 0.5488, 0.3192, 0.0988, 0.0198, ..., etc. The proportion of cells with the same number of asymmetrical exchanges would be the same, and the relative frequencies of the various combinations would be as shown in Table 3. We have assumed that all asymmetrical exchanges are lethal; thus, any cell containing one will never propagate, and we must contend only with those having only symmetrical exchanges. Thus, if we take a theoretical population of 1,000 scored cells, approximately 50% of which were in S at the time of the MMS treatment and sustained chromosome damage at the level of 120% chromatid interchanges, only 3.4% symmetrical exchanges would be expected to be seen in the subsequent primary spermatocytes.

This figure is derived in the following way. Half, or 500, of the 1000 cells would have been unlabeled and practically void of

Table 3. Expected Poisson distributions of chromatid exchange classes based on a mean yield of 60% symmetrical and 60% asymmetrical exchanges.

		Asymmetrical[a]				
		0	1	2	3	
Symmetrical		0.5488	0.3293	0.0988	0.0231	
0	0.5488	0.3012	0.1807	0.0542	0.0127	
		(151)	(90)	(27)	(6)	274
1	0.3293	0.1807	0.1084	0.0325	0.0076	
		(90)	(54)	(16)	(4)	164
2	0.0988	0.0542	0.0325	0.0098	0.0023	
		(27)	(16)	(5)	(1)	49
3	0.0231	0.0127	0.0076	0.0023	0.0005	
		(6)	(4)	(1)		11
		274	164	49	11	498

[a] The numbers in parentheses correspond to the numbers of cells in each class from a total population of 500 cells. The totals do not add to 500 cells due to rounding off.

exchanges. The sum total of 600 exchanges would have been in the labeled cells, and we assume that half, or 300, of them would have been asymmetrical and the other half symmetrical. We then assume that the exchange classes are distributed in a random fashion, so that the proportions of cells with 0, 1, 2, or 3 exchanges of each type are the same as those given for the respective classes in Table 3. The various proportions are then multiplied to determine what fractions would have zero asymmetrical exchanges and either 0, 1, 2, or 3 symmetrical exchanges. When this is done it is seen that of the original 500 cells that were labeled, 151 would have no exchanges, 90 would have a single symmetrical exchange, 27 would have two symmetrical exchanges, and 6 would have three symmetrical exchanges. All the rest would have at least one asymmetrical exchange and would be killed.

Next we assume that 20% of all the symmetrical exchanges are incomplete and cell lethal. Thus the 90 singles would be reduced to 72, the 27 doubles to 17 ($27 \times 0.8 \times 0.8$, since both exchanges must be complete to insure survival), and the six triples to three. Next we correct for random segregation at the first mitosis, and the new numbers of exchange-bearing cells become: single = 21 (18 from the original singles plus 2 from the original doubles plus approximately 1 from the original triples); double = 1; and triple < 1. This adds up to a total of 23 exchanges in 672 cells (500 unlabeled, 150 labeled zero class, 21 single class, 1 double). Thus the original yield of 60% chromatid exchanges observed in treated spermatogonia is expected to yield only 3.4% reciprocal translocations in the primary spermatocytes. The yield of 3.4% estimated in this example will, of course, vary if the values for labeling index or the proportion of incompleteness differ from those used here. These calculations do not take into account possible selective disadvantages caused by the translocation once it survives the first mitotic division. These could conceivably reduce the recovered yield even more. It is reasonably obvious from these calculations that restraint should be used in estimating genetic hazards from chromatid aberration yields in bone marrow and spermatogonia.

We hasten to add that our considerations are theoretical in nature, and that they should be tested by an experiment designed to enable the parameters used in the foregoing example to be determined. In this way, the proportion of reciprocal translocations expected to be found in primary spermatocytes from a known yield observed in spermatogonia could be compared with the actual number observed in spermatocytes after treatment of spermatogonia. The aberrations should be classified so that the frequencies of symmetrical and asymmetrical interchanges would be known; the proportion of incomplete interchanges should also be recorded; and the labeling indexes as well as the frequencies of aberrations in labeled and unlabeled cells should also be obtained. Such information would enable us to offer some explanation for the observation that certain chemicals induce chromatid aberrations in spermatogonial cells but none are recovered in primary spermatocytes, and it should also provide a basis for evaluating the possible genetic hazard from chemicals.

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